

# Angiotensin II causes beta cell dysfunction through an ER stress induced pro-inflammatory response

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## Abstract

The metabolic syndrome is associated with an increase in the activation of renin angiotensin system (RAS) and inhibition of RAS reduces the incidence of new onset diabetes. Importantly, angiotensin II (AngII), independently of its vasoconstrictor action causes beta-cell inflammation and dysfunction, which may be an early step in the development of type-2 diabetes. The aim of this study was to determine how AngII causes beta cell dysfunction. Islets of Langerhans were isolated from C57BL/6J mice that had been infused with AngII in the presence or absence of taurine-conjugated ursodeoxycholic acid (TUDCA) and effects on ER stress, inflammation and beta cell function determined. The mechanism of action of AngII was further investigated using isolated murine islets and clonal beta cells.

We show that AngII triggers ER stress, an increase in the mRNA expression of pro-inflammatory cytokines, and beta cell dysfunction in murine islets of Langerhans both *in vivo* and *ex vivo*. These effects were significantly attenuated by TUDCA, an inhibitor of ER stress. We also show that AngII-induced ER stress, is required for the increased expression of pro-inflammatory cytokines and, is caused by ROS and IP3 receptor activation.

These data reveal that the induction of ER stress is critical for AngII-induced beta cell dysfunction and indicates how therapies that promote ER homeostasis may be beneficial in the prevention of type-2 diabetes.

**Abbreviations:** AngII (angiotensin II), 2APB (2-aminoethoxydiphenyl borate), AT1R (angiotensin type 1 receptor), ATF4 (activating transcription factor 4), CHOP (C/EBP Homologous Protein), ER (endoplasmic reticulum), GK (glucokinase), IRE1 (inositol requiring enzyme 1), PERK (PKR-like ER kinase), RAS (renin angiotensin system), TUDCA (taurine-conjugated ursodeoxycholic acid), UPR (unfolded protein response),

## Introduction

Elevated blood pressure due to increased activation of the renin angiotensin system (RAS) is an important feature of the metabolic syndrome. This constitutes a series of metabolic disorders that increase the risk of developing type-2 diabetes and cardiovascular disease. Importantly, the pharmacological inhibition of RAS reduces the incidence of new onset type-2 diabetes in high risk populations (1–3) and RAS blockade in several animal models of diabetes improves pancreatic beta cell function (4–8). Conversely, the infusion of AngII into mice causes beta cell dysfunction (9–11). As the components of the RAS system have been detected in islets and this ‘local’ RAS plays an important role in regulating islet mass and function (7,9), the effects of RAS blockade *in vivo* is likely mediated by inhibiting locally produced AngII.

The detrimental effects of AngII on beta cell function were largely attributed to vasoconstriction resulting in decreased delivery of glucose to pancreatic beta cells (12–14). However, it has recently been shown that AngII infusion in mice causes beta cell dysfunction independently of AngII’s effect on blood pressure (11). In support of this, the treatment of either isolated human or rodent islets with AngII also causes beta cell dysfunction (9–11). The damaging effects of AngII on beta cell function, both *in vivo* and *in vitro*, have been ascribed to an increase in the expression of pro-inflammatory cytokines, in particular IL-1 $\beta$  (11), and there is a growing body of evidence indicating that inflammation is important in the development of beta cell dysfunction in type-2 diabetes (15–17). Endoplasmic reticulum (ER) stress is also associated with the loss of beta cell function and viability in type-2 diabetes (18–21). This stress is sensed by the ER transmembrane proteins: PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol requiring enzyme 1 $\alpha$  (IRE1) that activate an adaptive response called the unfolded protein response (UPR) (21–23). If the UPR is unable

to alleviate ER stress beta cell dysfunction and death can occur through the chronic activation of a UPR, which activates a number of pro-apoptotic and pro-inflammatory signaling pathways (16,23,24). Given that AngII also increases the expression of pro-inflammatory cytokines and causes beta cell dysfunction (11), we hypothesised that ER stress may play an important role in AngII-mediated beta cell inflammation and dysfunction.

## Materials and Methods

*Cell culture.* Mouse insulinoma 6 (MIN6) cells (25) were used between passages 25 and 35 at ~80% confluence and cultured as previously described (26).

*Islet isolation.* Pancreatic islets were isolated from adult C57BL/6J mice (Animal Resources Centre, Perth, Australia)). Briefly the pancreas was inflated by injecting 3 ml of RPMI 1640 (Invitrogen) containing 1mg/ml collagenase (Sigma-Aldrich, Australia) through the common pancreatic duct and the pancreas excised. Islets were then isolated as previously described (26).

*Animal experimentation.* All experiments were approved by the Animal Ethics Committee of RMIT University (#1504). Male C57BL/6J mice (10 weeks of age) obtained from the Animal Resources Centre (Perth, Australia) were kept at 22±1°C on a 12-h light/dark cycle. All mice were fed standard mouse chow and water ad libitum. After 1 week of acclimatization, the mice were randomly assigned to 3 groups: 1) sham (control mice infused with PBS, n=8); AngII (mice infused with human AngII, dissolved in sterile 1XPBS, at 416ng.kg<sup>-1</sup>.min<sup>-1</sup> using subcutaneous ALZET® mini-osmotic pumps (USA) for 2 weeks, n=8); or AT (mice infused with AngII with daily intra-peritoneal injection of TUDCA at 150 mg.kg<sup>-1</sup>.day<sup>-1</sup> for 2 weeks,

n=8). Human Ang II ( $\geq 93\%$  HPLC) was purchased from Sigma. The body weight and plasma glucose levels were recorded three times weekly during the experiment. Systolic blood pressure (SBP) was measured using the CODA tail-cuff blood pressure system (ADInstruments Pty Ltd., Australia).

For Glucose tolerance tests (GTT; 2.5 g glucose/kg BW, ip) mice were fasted for 5 h prior to blood samples being collected via the tail vein and blood glucose concentration determined using a glucometer (AccuCheck Proforma Nano; Roche, Victoria, Australia). For insulin measurements blood samples were centrifuged (2000 rpm, 2 min at 4°C) and the plasma insulin concentrations measured by ELISA (Linco Research, St. Louis, MO) in collaboration with the Department of Physiology, Monash University, Melbourne. Mice were euthanized with CO<sub>2</sub>. The disposition index, a composite measure of beta cell function (27), was also calculated using the following formula:  $\Delta I_{0-30} / \Delta G_{0-30} \times 1 / \text{fasting insulin}$ .

*Western-blotting and Immunohistochemistry.* SDS-PAGE and western blotting were performed as previously described (28) using antibodies against: BiP (BD Transduction Laboratories, USA), phospho-IRE1 $\alpha$  (Ser 724) (Abcam, USA), phospho-PERK (Thr 980), phospho-eIF2 $\alpha$  (Ser 51), CHOP, ATF4, and GAPDH (Cell Signaling Technology, USA). Immunohistochemistry was performed on fixed and paraffin embedded pancreatic sections using anti-CHOP, and Alexa Fluor 488 conjugated antibodies. All antibodies were used as per manufacturer's instructions.

*Transfection, RNA isolation and qPCR analyses.* Silencer® Select siRNAs against *Ire* (cat. no. S95857) and *Xbp* (cat. no. S76114) were purchased from Ambion®oligos (Thermo Scientific, USA). siRNA oligos were transfected using Lipofectamine® RNAiMAX (Thermo Scientific, USA) according to the manufacturer's protocol. Total RNA was isolated using the

ReliaPrep™ RNA Cell Miniprep System (Promega, USA). Reverse transcription was carried out using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK). Quantitative PCR was carried out using the SYBR® Green PCR Master Mix (Applied Biosystems, UK) using primers described in ESM Table 1. The gene expression from each sample was analysed in duplicate and normalized against the housekeeper 18S. All reactions were performed on the Rotor-Gene Q (Qiagen, USA). The results are expressed as relative gene expression using the  $\Delta C_t$  method (29).

*Glucose stimulated insulin secretion (GSIS).* Isolated islets were cultured overnight in RPMI 1640 medium supplemented with L-glutamine (20 mmol/l), and FBS (5%). GSIS was performed as previously described (28). Insulin ELISA was performed as described above.

*Quantification of superoxide levels* Superoxide levels were measured using L-012 (Tocris Bioscience, USA) enhanced chemiluminescence as previously described (30). MIN6 cells were plated on a 96-well Optiplate (PerkinElmer, Melbourne, Australia), incubated with 100  $\mu$ mol/L L-012, and luminescence measured using a BMG Clariostar plate reader (BMG Labtech, Melbourne, Australia).

*Statistical analysis* Data are expressed as mean  $\pm$  SE, unless otherwise stated. Data were analysed by one-way ANOVA followed by Tukey's post-hoc test for multiple comparison between means using Prism 6 (GraphPad Software, USA). Differences were considered statistically significant at  $p < 0.05$ .

## Results

*Angiotensin II induces ER stress and impairs beta cell function in mouse islets of Langerhans.*

To investigate the role of ER stress on AngII-dependent beta cell dysfunction, mouse islets of Langerhans were treated with AngII for 96 h in the presence or absence of TUDCA, a chemical chaperone that inhibits ER stress (31). AngII caused beta cell dysfunction as demonstrated by a marked increase of basal insulin secretion and significant decrease in the stimulatory index compared to control islets reflecting the loss of the glucose-stimulated insulin secretion (GSIS) (Fig. 1a and b). This correlated with a decrease in the mRNA expression of glucokinase (*Gk*) (Fig. 1c), whereas the mRNA expression of glucose transporter-2 (*Glut-2*) and pancreatic and duodenal homeobox 1 (*Pdx1*) were increased (Fig 1c). AngII treatment also significantly increased the expression of the pro-inflammatory cytokines interleukin 1 $\beta$  (*Il-1 $\beta$* ) and tumor necrosis factor  $\alpha$  (*Tnf- $\alpha$* ) (Fig. 1d). However, no significant changes in monocyte chemotactic protein 1 (*Mcp-1*) were detected. Importantly, AngII caused a marked increase in the expression of markers of ER stress (Fig. 1e), including immunoglobulin binding protein (*BiP/Grp78*), the spliced form of X-box binding protein 1 (*Xbp1s*), endoplasmic reticulum oxidoreductin 1 (*Ero1l*), peptidyl-prolyl cis-trans isomerase (*Fkbp11*), ER degradation enhancing  $\alpha$ -mannosidase-like protein (*Edem*), activating transcription factor 4 (*Atf4*) and the pro-apoptotic transcription factor C/EBP Homologous Protein (*Chop* also known as *Gadd153*) (32,33). The co-administration of TUDCA with AngII significantly restored beta cell function, as demonstrated by a reduction in basal insulin secretion (Fig. 1a), an improved stimulatory index (Fig. 1b) and the restoration of *glucokinase* expression to control levels (Fig 1c). TUDCA also significantly decreased the expression of the pro-inflammatory cytokines *Il-1 $\beta$*  and *Tnf- $\alpha$*  (Fig 1d) and all the markers of ER stress investigated (Fig. 1e). Thus AngII causes beta cell dysfunction, ER stress and inflammation in mouse islets of Langerhans and these effects occur independently of AngII's systemic vasoconstrictive effects. Moreover, as TUDCA counteracts

the effects of AngII it is likely that the deleterious effects of AngII treatment observed here are mediated through ER stress.

*ER stress precedes the induction of pro-inflammatory cytokines in AngII treated MIN6 cells and Islets of Langerhans.* We initially investigated the efficacy of using the pancreatic beta cell line MIN6 as a model to further investigate AngII-induced-ER stress and inflammation. MIN6 cells were treated with AngII or thapsigargin, a pharmacological inducer of ER stress, in the presence or absence of TUDCA (ESM Fig 1). As observed in islets, AngII treatment caused ER stress and an increase in the expression of *Il-1 $\beta$* , which was inhibited by TUDCA. Thapsigargin also evoked a UPR and increased the expression of *Il-1 $\beta$* .

To investigate the temporal relationship between AngII-induced ER stress and the expression of pro-inflammatory cytokines, MIN6 cells were treated with AngII for up to 96 h and the induction of ER stress and the expression of *Il-1 $\beta$* , *Tnf- $\alpha$*  and *Mcp-1* were monitored. AngII rapidly induced ER stress (within 2 h) as determined by the phosphorylation status of: IRE1 $\alpha$ , PERK, PERK's substrate eIF2 $\alpha$ , and an increase in the expression of BiP, ATF4 and CHOP and *Xbp1s*, (Fig. 2a and b). AngII increased the expression of thioredoxin interacting protein (TXNIP) (16) (Fig. 2b) at 6h and the expression of the pro-inflammatory cytokine *Il-1 $\beta$*  and *Tnf- $\alpha$*  mRNA by 6 h and 48 h respectively (Fig. 2c). No changes in the expression of *Mcp1* were detected (Fig. 2c). AngII treatment of mouse islets caused an increased in the expression of *Xbp1s*, *Atf4* and *Chop* which preceded an increase in the expression of *Il-1 $\beta$*  and *Tnf $\alpha$*  (Fig 2d).

Therefore, the occurrence of ER stress precedes an increase in the expression of the pro-inflammatory cytokines providing evidence that AngII-induced ER stress may promote inflammation in beta cells.



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199 *The role of PERK and IRE1 $\alpha$  in AngII-induced expression of the pro-inflammatory cytokines.*

200 To determine how ER stress increases the expression of the pro-inflammatory cytokines, we  
201 investigated the effect of a selective inhibitor of PERK (GSK2606414 (PERKi)) and siRNA  
202 mediated knock-down of *Irel $\alpha$*  or *Xbp1* on AngII-induced *Il-1 $\beta$*  expression. GSK2606414  
203 inhibited AngII-induced phosphorylation of eIF2 $\alpha$  (Fig. 3a) and expression of ATF4, CHOP  
204 (Fig. 3a), *Txnip* (Fig. 3b) and importantly *Il-1 $\beta$*  (Fig. 3b). As anticipated siRNAs directed  
205 towards *Irel $\alpha$*  or *Xbp1* significantly reduced *Xbp1s* basal expression and AngII induced  
206 increases in *Xbp1s* expression (Fig. 3c). Importantly, siRNA-mediated knock-down of *Irel $\alpha$*   
207 or *Xbp1* also inhibited AngII evoked increases in *Txnip* and *Il-1 $\beta$*  expression (Fig. 3d). These  
208 results provide evidence that both PERK and IRE1 $\alpha$  are required for Ang II to induce a pro-  
209 inflammatory response.

210

211 *Angiotensin-II induced ER stress is dependent upon both IP3R and NOX activation. AngII-*  
212 *induced ER stress and inflammation is dependent on AT1R activation as irbesartan (IRB), an*  
213 *angiotensin 1 receptor (AT1R) antagonist, attenuated both AngII-induced ER stress and Il-1 $\beta$*   
214 *expression (ESM Fig. 2). The AT1R classically couples to Gq/11 and activates NADPH*  
215 *oxidase (NOX) and phospholipase-C (PLC) resulting in an increase in IP3 and ROS (34,35).*  
216 *IP3 stimulates ER calcium release (34) and ROS has been shown to sensitise the IP3 receptor*  
217 *(IP3R) (36). As a decrease in ER calcium can induce ER stress (26) we investigated whether*  
218 *the effects of AngII were mediated by an IP3R-dependent mechanism. MIN6 cells were treated*  
219 *with AngII in the presence or absence of selective IP3 receptor antagonists, 2-*  
220 *aminoethoxydiphenyl borate (2APB) and xestospongine-C (XestC). 2APB and Xest-C inhibited*  
221 *AngII-induced ER stress as determined by a significant decrease in eIF2 $\alpha$  phosphorylation, as*

well as the expression of ATF4 and CHOP (Fig. 4a). Therefore, AngII-induced ER stress requires IP3R activation indicating that AngII-induced ER stress is likely to be mediated by a decrease in ER calcium. However, for reasons which are unclear, at 6h treatment of cells with AngII in the presence of xestospongine-C potentiated AngII-induced eIF2 $\alpha$  phosphorylation (Fig. 4a).

To investigate the role of ROS, changes in superoxide production in MIN6 cells in response to AngII in the presence or absence of the AT1R antagonist irbesartan, and two selective inhibitors of NOX, apocynin and diphenyleneiodonium (DPI) was determined (Fig. 4b). As anticipated AngII increased superoxide levels and this was inhibited by apocynin (at 10 and 300  $\mu$ M), DPI and irbesartan (Fig. 4b). Importantly, apocynin or DPI also inhibited AngII-induced ER stress as determined by the phosphorylation of eIF2 $\alpha$  and the expression of ATF4 and CHOP (Fig. 4c). These data provide evidence that ROS is required for AngII-induced ER stress. Given that IP3R activation is also required, it is possible that ROS promotes ER stress by sensitizing the IP3R (36).

*High glucose potentiates AngII-induced ER stress.* To investigate the effect of glucose concentration on AngII-induced ER stress. MIN6 cells were incubated at either low (5.5mM) glucose or high (25mM) glucose and the effect of AngII on ER stress determined. AngII treatment of MIN6 cells incubated at low glucose caused a significant increase in the expression of CHOP and ATF4 which marks the presence of ER stress. Interestingly, incubation at high glucose (25mM) potentiated the effect of AngII on ER stress (ESM Fig 3a). These experiments were repeated using isolated murine islets and similar results were obtained (ESM Fig 3b). Thus, high glucose potentiates the effects of AngII on ER stress.

Angiotensin II infusion of mice causes ER stress and an increase in the expression of pro-inflammatory cytokines in pancreatic islets. To assess whether a chronic elevation in AngII caused ER stress in islets *in vivo* and whether this was important in the induction of pro-inflammatory cytokines, mice were infused with AngII for 2 weeks with or without the co-administration of TUDCA. Following AngII infusion, mice displayed a marked impairment of glucose tolerance (Fig. 5a, b) together with elevated levels of fasting plasma insulin (Fig. 5c), likely due to the known detrimental effects of AngII on insulin sensitivity (37). Interestingly, there was also evidence of beta cell dysfunction as determined by a decrease in the disposition index (Fig. 5d). The co-administration of TUDCA improved glucose tolerance, reduced plasma insulin levels (Fig. 5a and c) and rescued beta cell function (Fig. 5d). All these effects occurred independently of changes in body weight, adiposity, or a sustained increase in systolic blood pressure (SBP; ESM Fig 4). Importantly, islets isolated from these AngII infused animals showed signs of ER stress as evidenced by an increase in the expression of *Xbp1s*, *Fkbp11*, *Ero1l*, *Edem*, *Atf4* and *Chop* and *Txnip* (Fig. 5e). Although no change in the expression of *BiP*, an adaptive marker of the UPR, was detected (Fig. 5e). Importantly, *Il-1 $\beta$*  and *Tnf- $\alpha$* , expression were also augmented by AngII infusion (Fig. 5f). Interestingly, as observed in isolated islets, AngII caused an increase in *Pdx1* and *Glut2* expression but a decrease in *glucokinase* expression (Fig. 5g). The co-administration of TUDCA inhibited AngII-stimulated increase in the expression of markers of ER stress (Fig. 5e), the pro-inflammatory cytokines (Fig. 5f) and the markers of beta-cell function (Fig. 5g). Taken together, these data provide evidence that *in vivo* AngII causes ER stress and that this increases the production of pro-inflammatory cytokines in islets.

To determine whether macrophages were present in islets isolated from AngII treated mice we looked for the presence of *F4/80*, a macrophage specific marker, by qPCR (ESM Fig. 5).

Although *F4/80 mRNA* was detected in islets its expression was unchanged by AngII indicating the macrophages are unlikely to be the source of the pro-inflammatory cytokines.

## Discussion

This study provides strong evidence that AngII causes ER stress to beta cells/islets both *in vitro* and *in vivo* and that this results in a pro-inflammatory phenotype and beta cell dysfunction. In addition, we provide a novel insight into how AngII causes ER stress (Fig. 6). We show that at high glucose AngII, via AT1R activation, promotes IP3R activation and an increase in ROS. This, likely via a decrease in ER calcium, results in ER stress and an increase in the expression of pro-inflammatory cytokines mediated by the activation of PERK and IRE1 $\alpha$ .

Although inflammation and the UPR are protective responses, chronic inflammation and/or UPR activation is associated with the pathogenesis of many diseases including type-2 diabetes (23). Indeed chronic inflammation has been implicated in beta cell dysfunction in type-2 diabetes and treatment with either anakinra, an IL-1 receptor antagonist, or anti IL-1 $\beta$  antibodies improves beta cell function, improves glycemic control and reduces inflammation (38–40). Moreover, IL-1 $\beta$  antagonism protects against the deleterious effects of AngII on islet function in HFD fed mice (11). Thus inflammation is a key mediator of AngII-induced beta cell dysfunction (11). A role for ER stress in AngII-mediated inflammation has recently been shown in other cell types/tissues (41–43), yet the mechanism by which this occurs had not been fully explored.

In this study we demonstrate that AngII-stimulated increase in pro-inflammatory cytokine expression in islets and beta cells is caused by ER stress as: 1) *Il-1 $\beta$*  and *Tnf- $\alpha$*  expression is inhibited by TUDCA; 2) *Il-1 $\beta$*  expression is inhibited by inhibition/reduced expression of

292 PERK and IRE1 $\alpha$ /XBP1 ; 3) ER stress precedes an increase in the expression of *Il-1 $\beta$*  and *Tnf-*  
293  *$\alpha$*  and; 4) thapsigargin increases the expression of *Il-1 $\beta$*  and *Tnf- $\alpha$* . This ER stress-induced  
294 increase in cytokine expression may be mediated by TXNIP promoted by the activation of  
295 PERK and IRE1 $\alpha$  as: 1) pharmacologically induced ER stress increases *Il-1 $\beta$*  mRNA  
296 expression through an increase in TXNIP expression, stimulated by the activation of PERK  
297 and IRE1 (16,44); 2) AngII increases *Txnip* expression via a PERK- and IRE1 $\alpha$ - dependent  
298 mechanism (Fig. 3) and; 3) *Txnip* precedes the expression of *Tnf- $\alpha$*  and *Il-1 $\beta$*  and is inhibited  
299 by TUDCA AngII induced ER stress is prevented by IP3R inhibition (Fig. 4). Intriguingly,  
300 inhibitors of NOX or the IP3R inhibit AngII-induced ER stress. As ER calcium depletion is  
301 known to cause ER stress (26,45–48) and ROS can potentiate IP3-dependent calcium release  
302 (36), ER calcium depletion is the likely cause of AngII-induced ER stress.

303 We show that chronic AngII treatment causes a decrease in beta cell function both *in vitro* and  
304 *in vivo* as evaluated by a loss of GSIS caused by an increase in the release of insulin at a lower  
305 threshold of glucose (Fig. 1 and 5). Similar results have been observed in rodent and human  
306 islets chronically treated with AngII and in HFD fed mice infused with AngII (11).  
307 Interestingly, the loss of GSIS observed in this study was associated with decreased  
308 glucokinase expression (Fig. 1 and 6), a protein which sets the threshold for GSIS (49,50). As  
309 the expression of glucokinase and GLUT2 are positively related to the state of differentiation  
310 of the beta cells and their expression is stimulated by PDX1 (51–53), it is surprising that AngII  
311 increases *Pdx1* and *Glut2* expression both *in vitro* and *in vivo* (Fig. 1 and 6). However, we  
312 detected no change in PDX1 protein expression (results not shown). Thus the significance of  
313 these changes in mRNA expression are unclear.

314 The components of the RAS system are expressed in islets and these are up-regulated in animal  
315 models of diabetes. Thus locally generated AngII rather than systemic AngII likely play an

important role in islet inflammation and ultimately beta cell dysfunction in type-2 diabetes (7,9,54). Interestingly, the AT1R antagonist losartan reduces high glucose induced ER stress and decreased beta cell function in human islets (55). Moreover, the administration of losartan to *db/db* mice improves islet function and mass, delays the onset of diabetes yet, has no effect on insulin sensitivity (7). Thus high glucose induced ER stress and beta cell dysfunction is, at least in part, mediated by AngII acting via the AT1R. Interestingly, we found that high glucose potentiated the effects of AngII on ER stress in MIN6 cells and murine islets. In addition, AngII exacerbates palmitate-induced ER stress in MIN6 cells (unpublished results). Therefore hyperglycemia and/or obesity may potentiate the deleterious effect of increased local AngII on beta cell function by exacerbating ER stress.

Together, these findings provide evidence that ER stress is a critical link between AngII and the induction of pro-inflammatory cytokines and that this may represent an initiating and/or early step in the development of beta cell dysfunction in type-2 diabetes.

## Acknowledgments

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## Figure legends

**Figure 1. Angiotensin II evokes ER stress and impairs beta cell function in mouse islets of Langerhans.** Mouse islets of Langerhans were treated with AngII (1μmol/l) (AngII) in the absence or presence of TUDCA (500 μg/ml) (AngII plus TUDCA = A+T) for 4 days prior to: (a) performing a GSIS assay to determine (b) the stimulatory index or (c-e) qPCR analyses of: (c) markers of beta cell function (*glucokinase (Gk); Glut2 and Pdx1*); (d) pro-inflammatory cytokines (*Il-1β, Tnf-α, and Mcp-1*); (e) markers of ER stress (*BiP, Xbp1s, Ero1l, Fkbp11, Edem, Atf4, Chop*); The results are expressed as the mean +/- S.E.M of three independent experiments. \*  $p < 0.05$ , \*\*  $p < 0.01$  vs control; ††  $p < 0.01$  for the compared groups.

**Figure 2. ER stress precedes the expression of pro-inflammatory cytokines.** MIN6 cells were treated for up to 96 h with 1μmol/l AngII. (a) Western-blot analysis of BiP, ATF4, CHOP and the phosphorylated form of IRE1α (p-IRE1), PERK (p-PERK) and eIF2α (p-eIF2α).

GAPDH was used as a loading control. Densitometric analyses of the results are presented below. qPCR analyses of: **(b)** markers of ER stress (*Xbp1s*, *Chop*, *Tnxip*) and; **(c)** pro-inflammatory cytokines (*Il-1 $\beta$* , *Tnf- $\alpha$*  and *Mcp-1*). **(d)** Mouse islets were treated for up to 42 h with 1 $\mu$ mol/l AngII. qPCR analyses of markers of ER stress (*Xbp1s*, *Atf4*, *Chop*, *Tnxip*) and pro-inflammatory cytokines (*Il-1 $\beta$*  and *Tnf- $\alpha$* ). The results are expressed as mean  $\pm$  S.E.M of four independent experiments. \*  $p < 0.05$ , \*\*  $p < 0.01$  vs control.

**Figure 3. Role of PERK and IRE1 in Angiotensin II induced expression of *Il-1 $\beta$* .** MIN6 cells were treated with 1 $\mu$ mol/l AngII for 6 h in the presence or absence of vehicle (DMSO), 0.5  $\mu$ mol/l GSK2606414 (PERKi) **(a)** Western-blot analysis of BiP, ATF4, CHOP phospho-IRE1 $\alpha$  (p-IRE1 $\alpha$ ), phospho-PERK (p-PERK) and phospho-eIF2 $\alpha$  (p-eIF2 $\alpha$ ). GAPDH was used as a loading control. Densitometric analyses of the results are presented below. **(b)** qPCR analyses of *Tnxip*, *Il-1 $\beta$*  and *Xbp1s*. The results are expressed as mean  $\pm$  S.E.M of three independent experiments. \*  $p < 0.05$ , \*\*  $p < 0.01$  vs their control; ♦♦  $p < 0.01$  vs the control group of siCon. MIN6 cells were transfected with control siRNA or siRNA against *Ire* or *Xbp*. 96 h post transfection cells were treated with 1 $\mu$ mol/l AngII for 6 h prior to qPCR analyses of **(c)** *Xbp1s*, *Ire1 $\alpha$*  and **(d)** *Tnxip*, and *Il-1 $\beta$* .

**Figure 4. IP3R and NOX activation is required for angiotensin II induced ER stress.** **(a)** MIN6 cells were treated with 1 $\mu$ mol/l AngII for 2 ~~or 6 h~~ in the presence of 2-aminoethoxydiphenyl borate (2APB) or xestospongine-C (XestC) (10 $\mu$ mol/l). Western-blot analysis of BiP, ATF4, CHOP, phospho-IRE1 $\alpha$  (p-IRE1 $\alpha$ ) and phospho-eIF2 $\alpha$  (p-eIF2 $\alpha$ ). GAPDH was used as a loading control. Densitometric analyses of the results are presented below. The results are expressed as mean  $\pm$  S.E.M of three independent experiments. \*

$p<0.05$ , \*\*  $p<0.01$  vs control; #  $p<0.05$  for the compared groups. (b) MIN6 were treated with 1  $\mu\text{mol/l}$  AngII for 6 h in the presence or absence of: 100 nmol/l irbesartan (IRB); 1 (+), 10 (++) or 300  $\mu\text{mol/l}$  (+++). apocycin (Apo) or; 10  $\mu\text{mol/l}$  diphenyleneiodonium (DPI). Superoxide levels were measured using L-012 enhanced chemiluminescence. The results are expressed as mean  $\pm$  S.E.M (NS (not significant)), \*\*  $p<0.01$ , \*  $p<0.05$ ; vs AngII treated and ††  $p<0.01$  vs control). (c) Representative western-blot of BiP, ATF4, CHOP, phospho-IRE1 and phospho-eIF2 $\alpha$ . GAPDH was used as a loading control. DPI and Apo used was 10  $\mu\text{mol/l}$ .

**Figure 5. Angiotensin II infusion in mice causes beta cell dysfunction, ER stress and increase in the expression of pro-inflammatory cytokines.** C57BL/6J male mice were infused with AngII (at 416 ng.kg<sup>-1</sup>.min<sup>-1</sup>) for 2 weeks. Where indicated TUDCA (150 mg.kg<sup>-1</sup>.day<sup>-1</sup>) was also administered (Sham (●), AngII (■), AngII plus TUDCA (A+T) / (▣)). (a) A GTT was conducted after 2 weeks of infusion and (b) the incremental area under the curve (iAUC) and (c) plasma insulin levels determined. (d) Disposition index is expressed as median  $\pm$  interquartile range. The results are expressed as mean  $\pm$  S.E.M. with eight mice per group unless otherwise stated. \*  $p<0.05$ , \*\*  $p<0.01$  vs sham; ††  $p<0.01$  vs AngII. Pancreatic islets were then isolated and mRNA expression of: (e) markers of ER stress; (f) pro-inflammatory cytokines and; (g) genes related to beta cell function were determined by qPCR analysis. The results are expressed as mean  $\pm$  S.E.M. with four mice per group. \*  $p<0.05$ , \*\*  $p<0.01$  vs control; †  $p<0.05$ , ††  $p<0.01$  for the compared groups.

**Figure 7. Schematic showing how angiotensin II causes inflammation in beta cells.** AngII binds to the AT1R resulting in the activation of G $\alpha$ q/PLC $\beta$  and NOX. This increases the production of ROS and IP3. ROS possibly sensitizes the IP3R for subsequent IP3-dependent



568 calcium release from the ER. This causes ER stress and the activation of the UPR, which  
569 promotes an increase in the expression of pro-inflammatory cytokines.

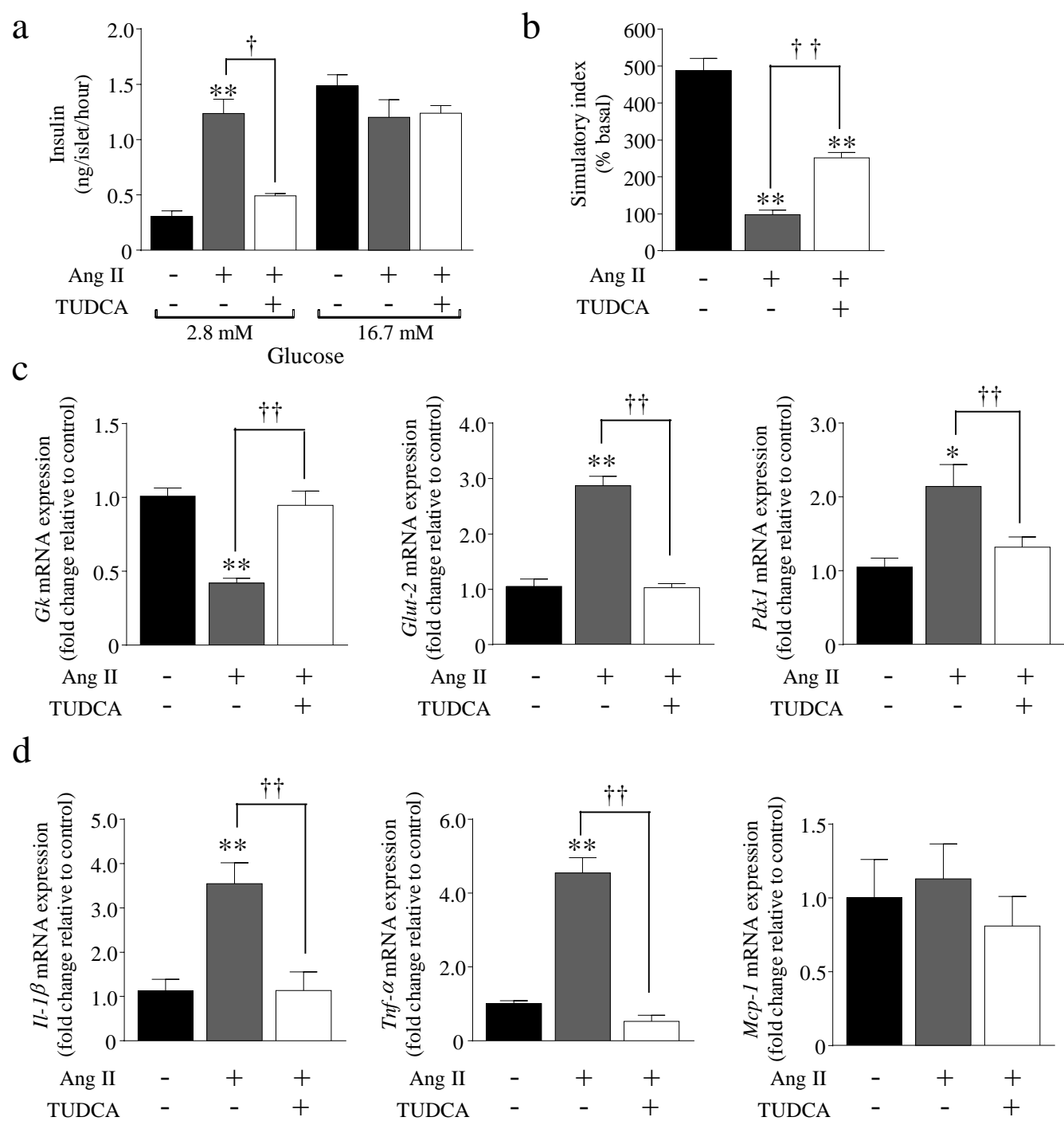


Figure 1

e

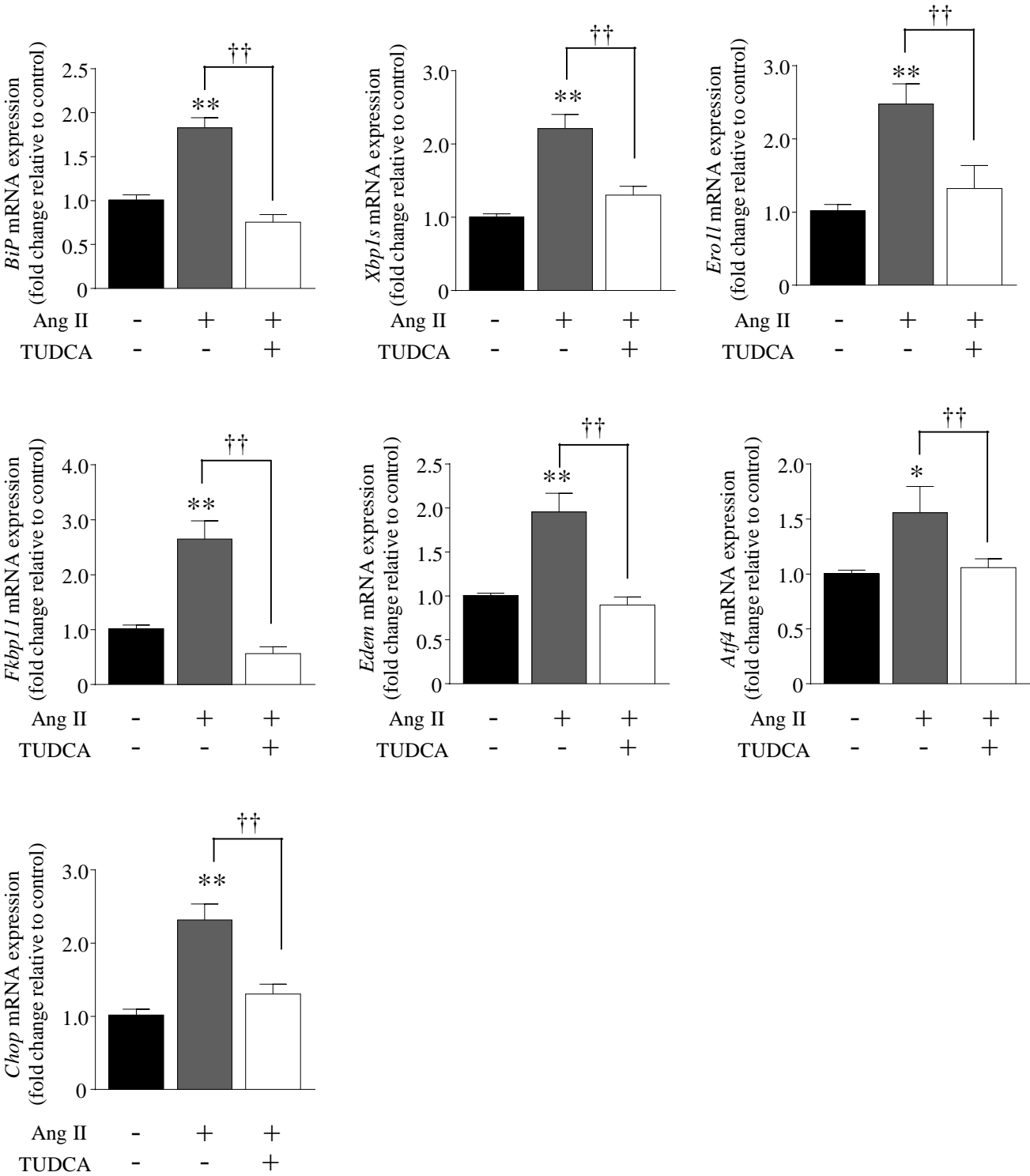
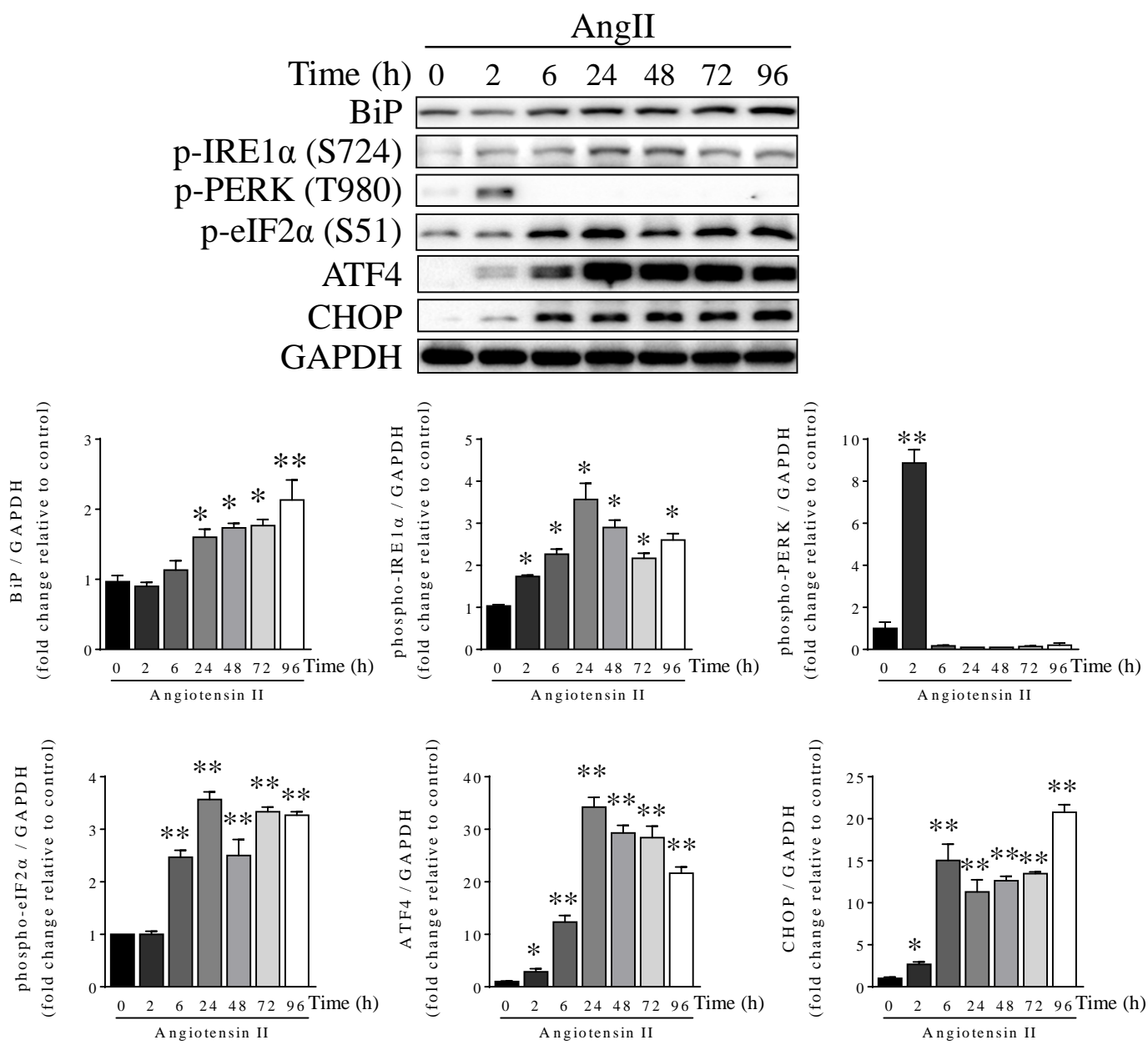


Figure 1 continued

a



b

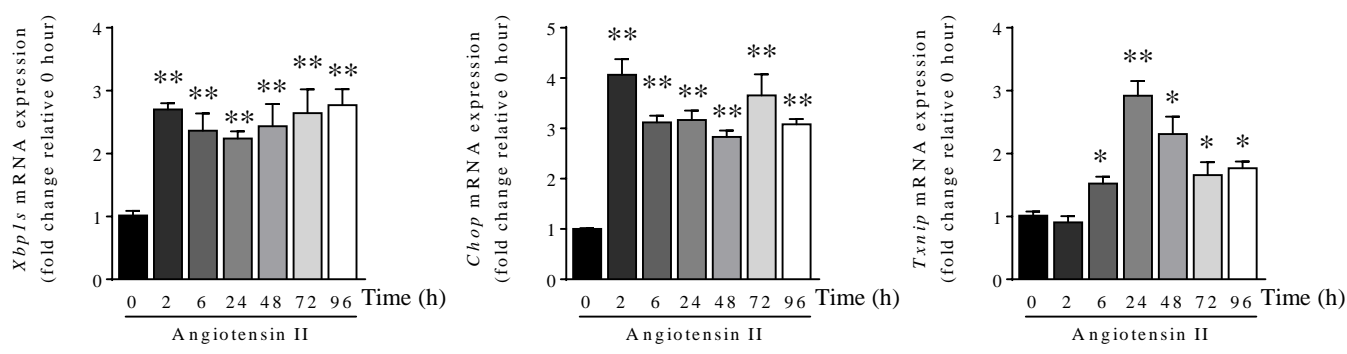
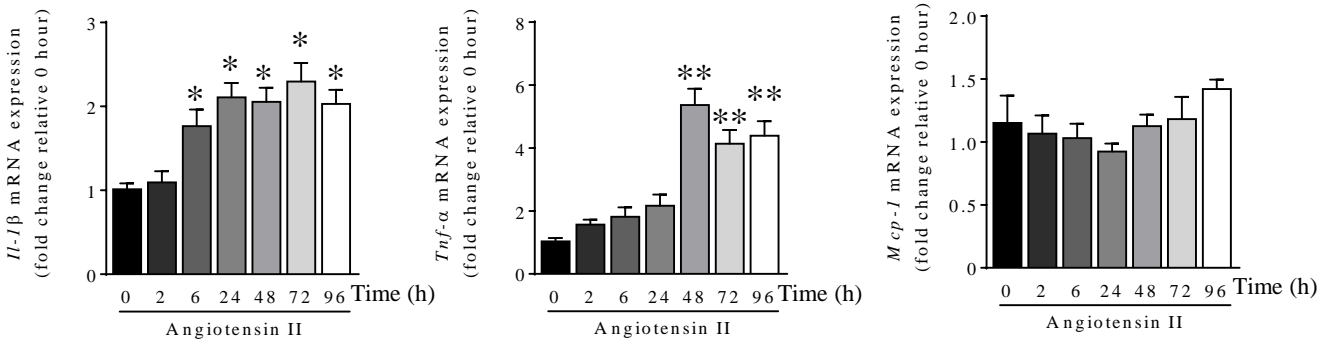


Figure 2

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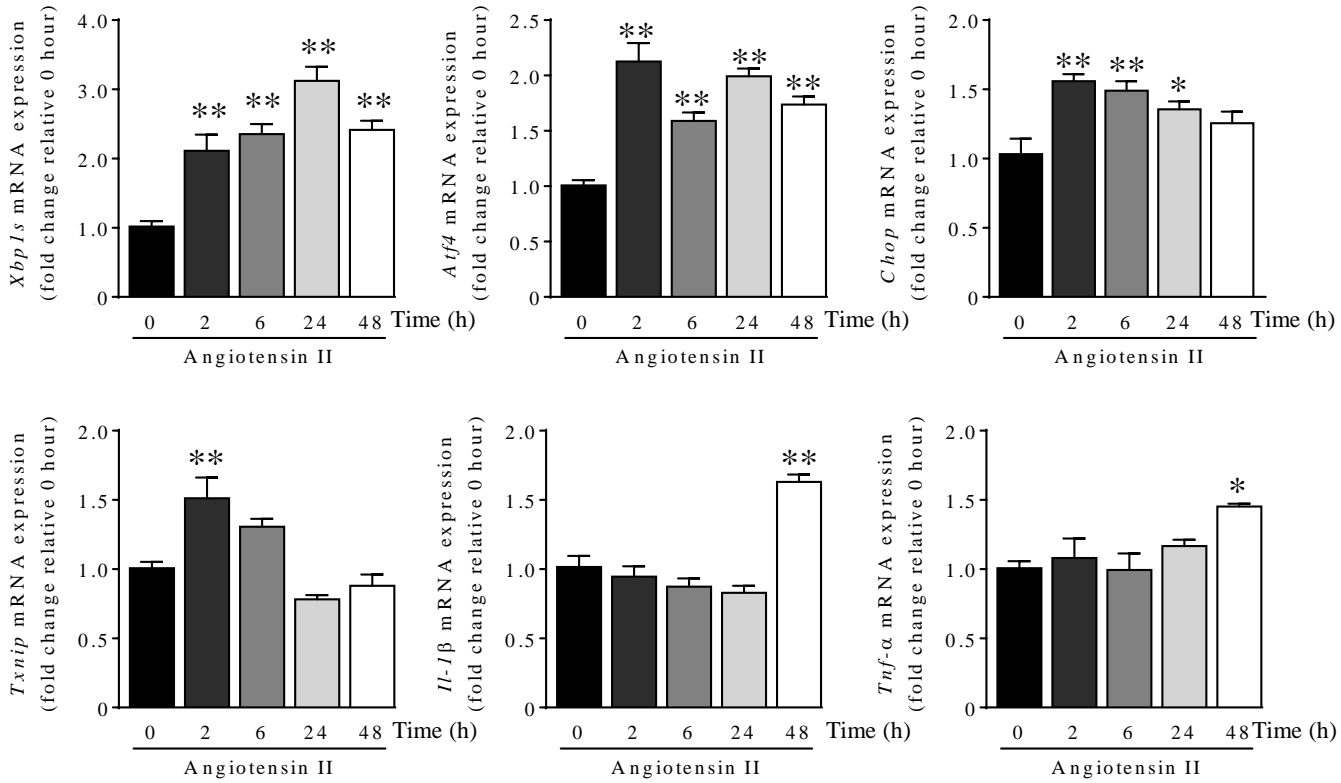
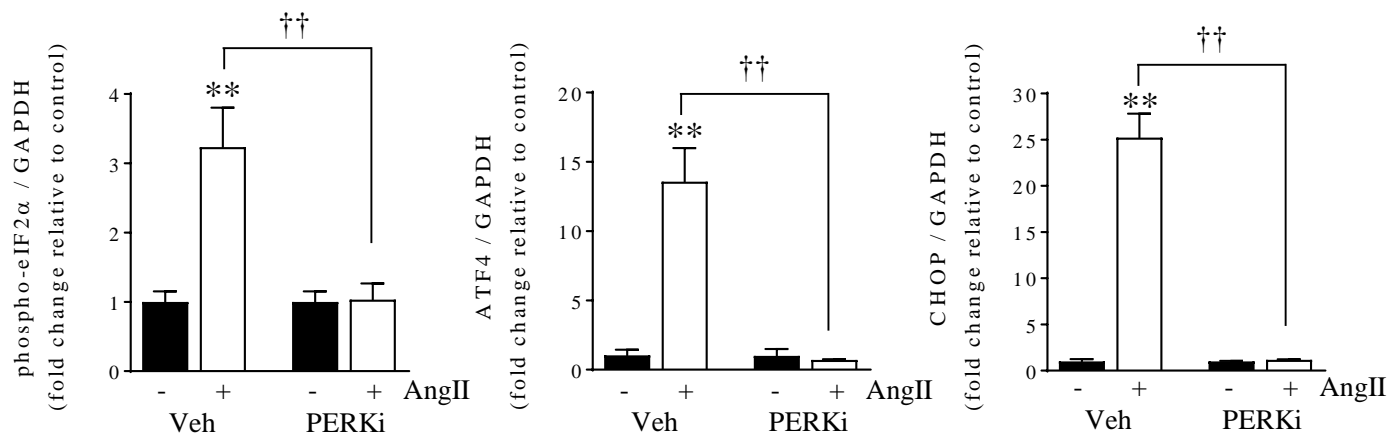
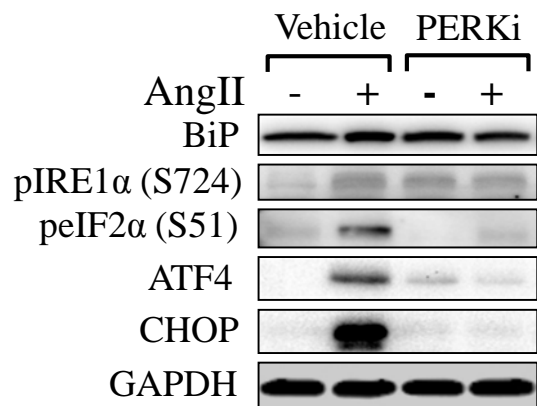
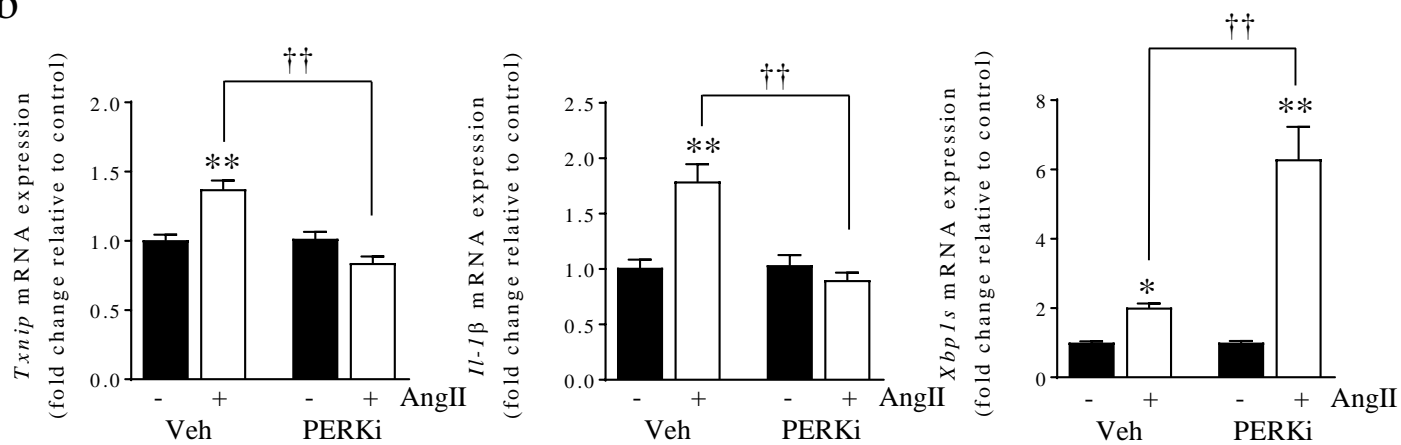


Figure 2 continued

a



b



c

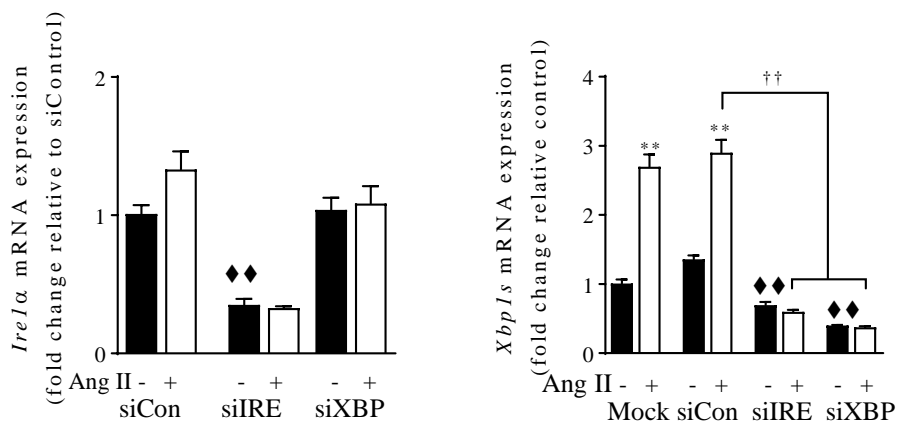


Figure 3

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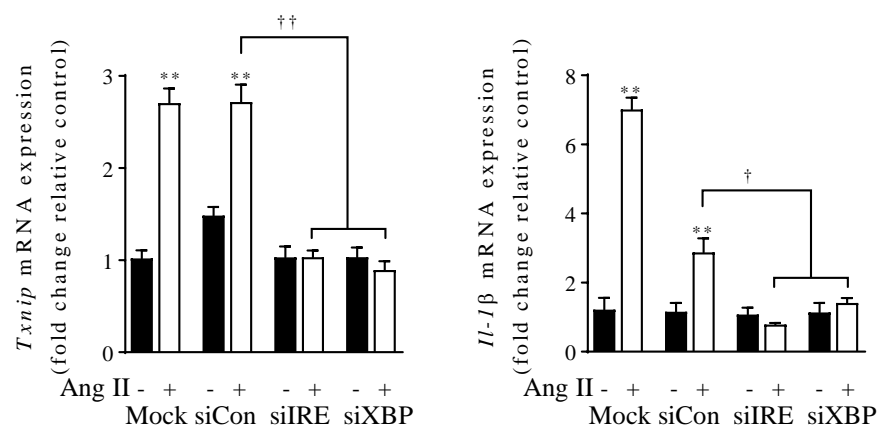


Figure 3 continued

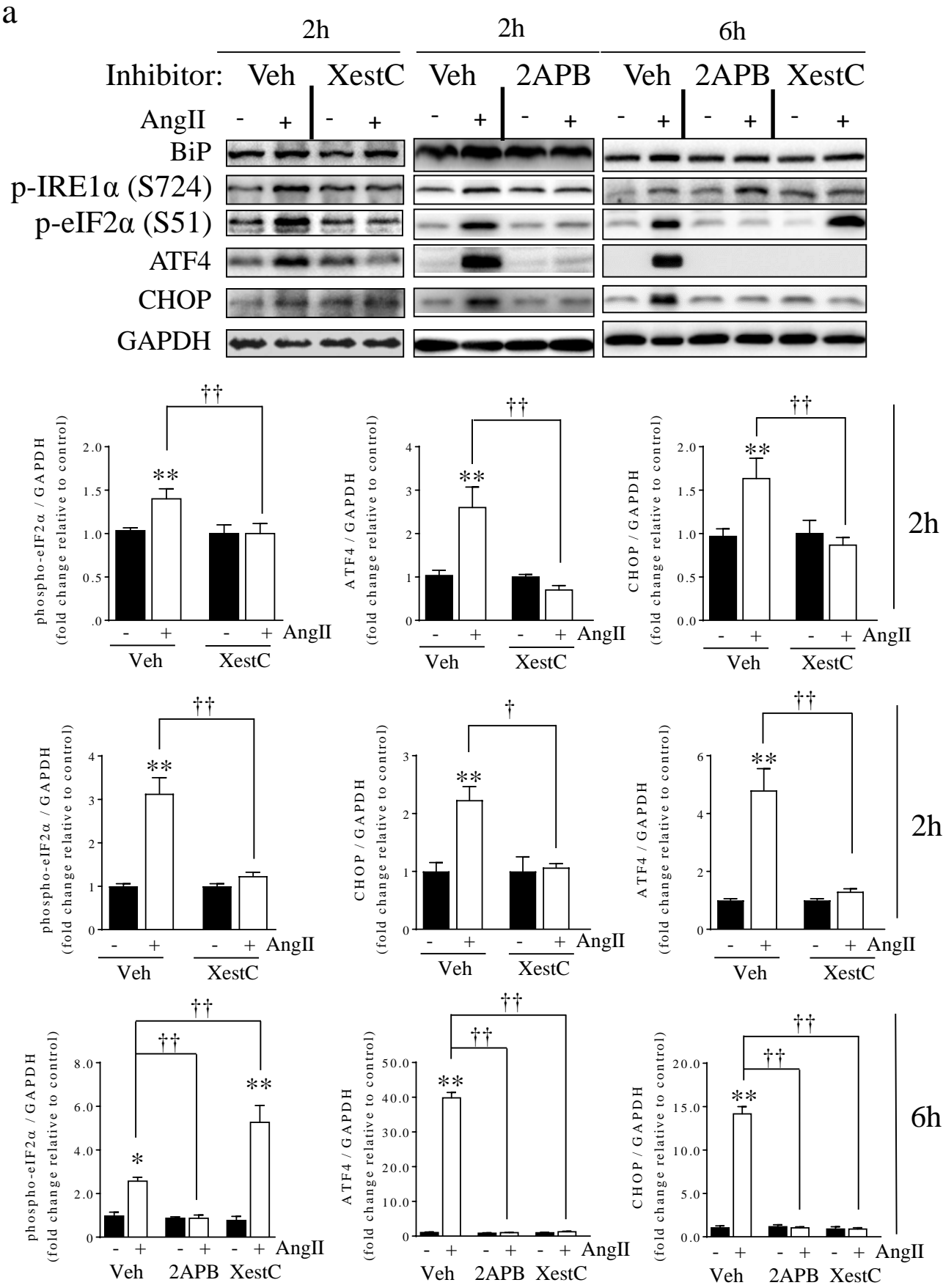


Figure 4



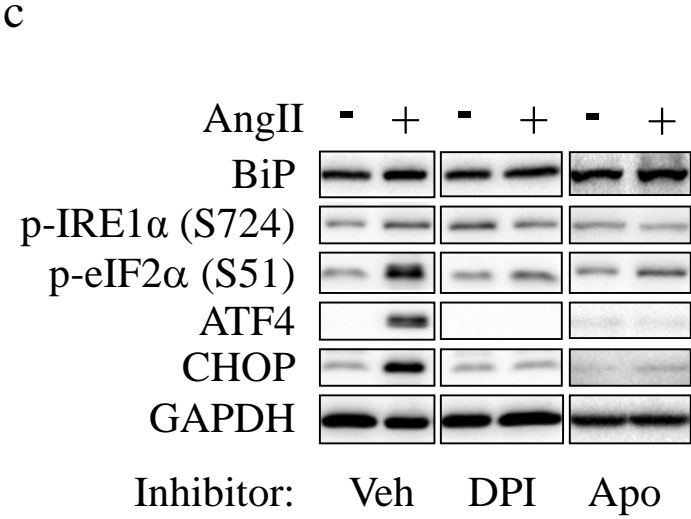
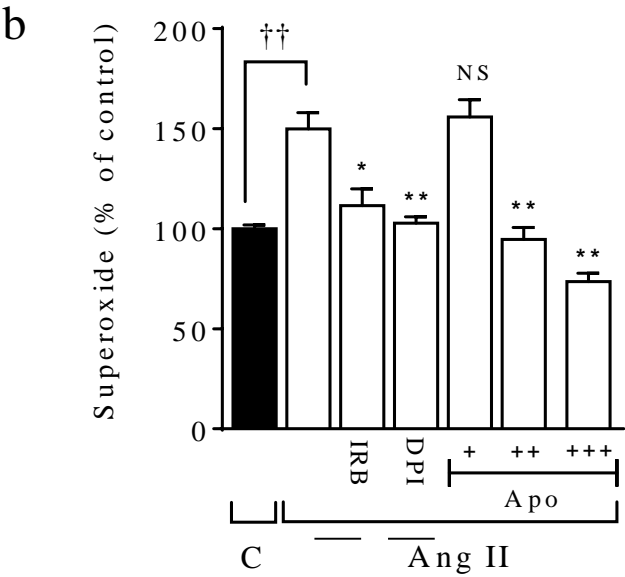


Figure 4 continued

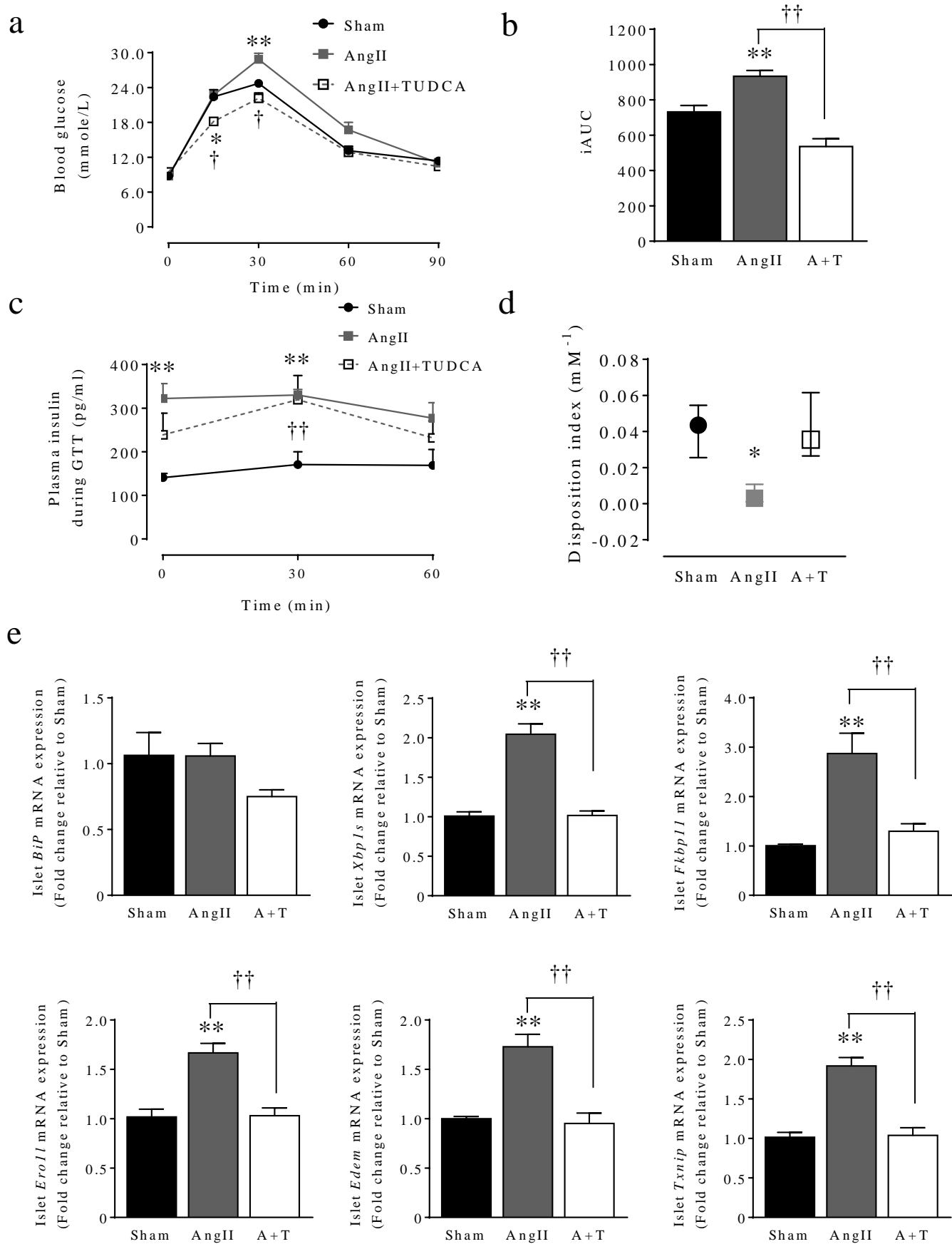
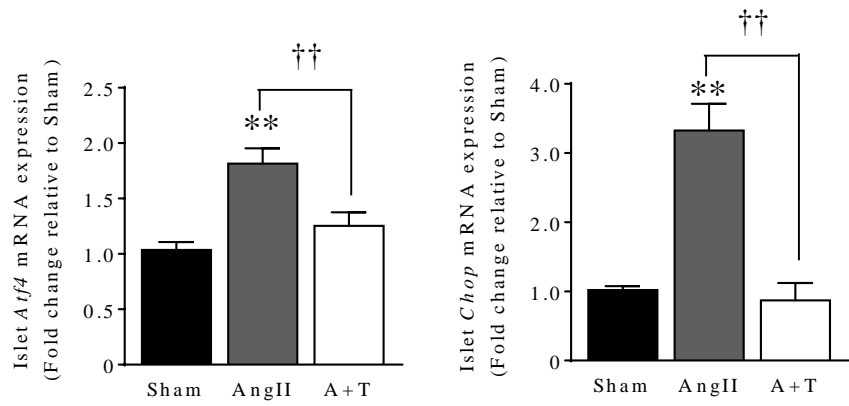
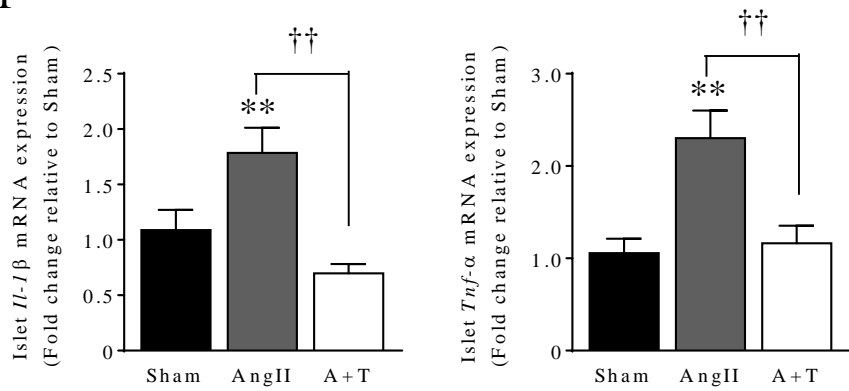


Figure 5

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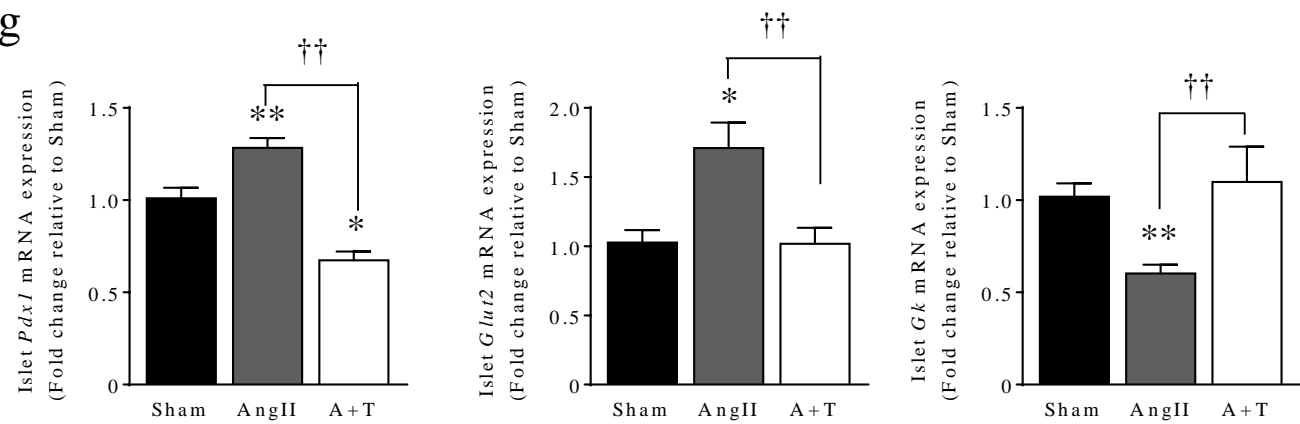


Figure 5 continued

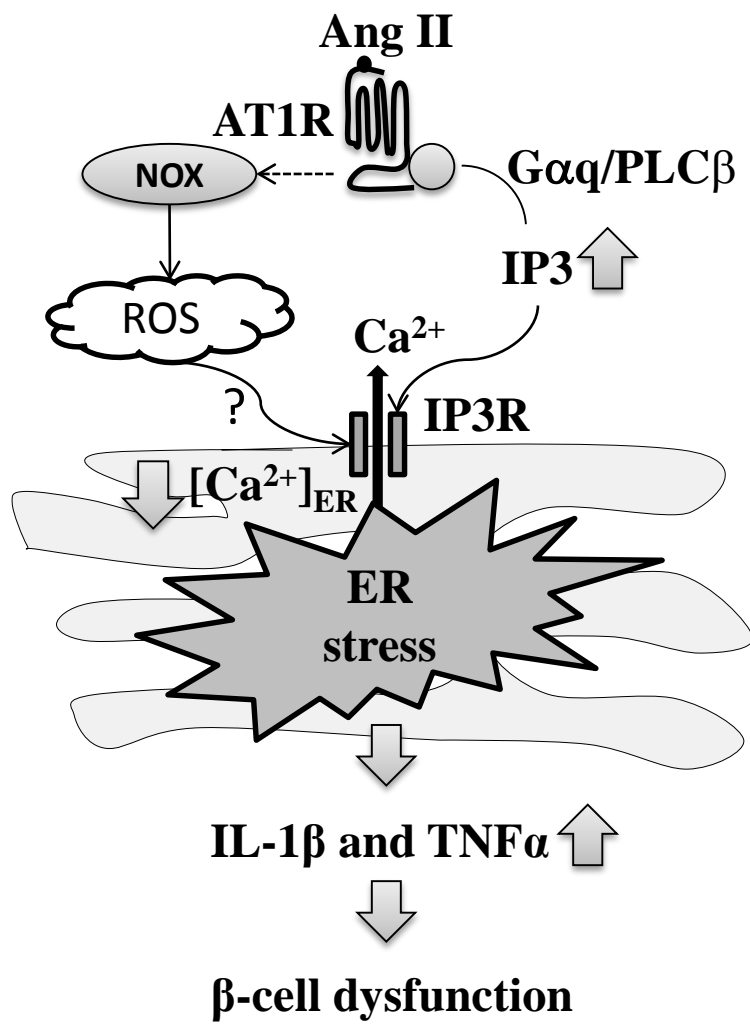


Figure 6